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Human Papillomavirus Type 18 E6 and E7 Antibodies in Human Sera: Increased Anti-E7 Prevalence in Cervical Cancer Patients

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Antibody-reactive regions on the human papillomavirus type 18 (HPV-18) E6 and E7 proteins were identified with rabbit polyclonal anti-fusion protein sera by screening of an fd phage expression library containing subgenomic HPV-18 DNA fragments and by testing of overlapping decapeptides representing the E6 and E7 open reading frames. Peptides comprising the delineated regions (designated E6/1 to E6/4 and E7/1) were synthesized and used in an enzyme-linked immunosorbent assay (ELISA) to detect anti-HPV-18 antibodies in human sera. A total of 232 human serum samples (identical numbers of cervical cancer patients and age-matched controls) collected in Tanzania were tested. Similar prevalences (between 0.8 and 4.3%) of antibodies recognizing the different E6 peptides were found in the sera from tumor patients and controls. With a synthetic 28-mer peptide (designated pepE701) comprising the E7/1 region, a significant difference was found: 10 of 116 tumor serum samples but 0 of 116 control serum samples showed a specific reaction ($P < 0.001$). This observation confirms earlier results with HPV-16 E7 fusion proteins (I. Jochmus-Kudielka, A. Schneider, R. Braun, R. Kimmig, U. Koldovsky, K. E. Schneweis, K. Seedorf, and L. Gissmann, J. Natl. Cancer Inst. 81:1698–1704, 1989). A lower prevalence of anti-HPV-18 E7 antibodies was observed when 188 human serum samples collected in Germany from tumor patients and controls were tested (3 of 94 positive in the cancer group; 0 of 94 positive in the control group). The type specificity of anti-HPV-18 E7 antibodies was demonstrated when the HPV type found by Southern hybridization in the cervical cancer biopsies was compared with seroreactivity: 4 of 8 serum samples obtained from HPV-18 DNA-positive but 0 of 16 serum samples from HPV-18 DNA-negative tumor patients reacted in the HPV-18 E7 ELISA. In addition, HPV-18-positive sera failed to react in a peptide ELISA with the homologous HPV-16 E7 region (M. Müller, H. Gausepohl, G. de Martinoff, R. Frank, R. Brasseur, and L. Gissmann, J. Gen. Virol. 71:2709–2717, 1990) and vice versa.

The genome of human papillomavirus type 18 (HPV-18) is found in cervical cancer biopsies at a frequency of about 3 to 20%, depending on the origin of the samples. In specimens obtained from Africa and Brazil and from black U.S. women the frequency of HPV-18-positive samples is significantly higher than it is in European patients (2, 5, 7, 12, 20, 26, 39). In all studies reported so far, the rate of HPV-18-positive cervical cancer biopsies is about 5 to 20 times lower than the proportion of tumors containing HPV-16 DNA. In contrast, comparable numbers of cervical cancer cell lines contain HPV-16 and HPV-18 genomes, indicating different biological properties of the two viruses (2, 36, 43). In fact, HPV-18 is more strongly associated with adenocarcinomas or adenosquamous carcinomas than with pure squamous cell carcinomas (25, 38, 41), and there is evidence for a more aggressive behavior and a higher recurrence rate of HPV-18-positive cancers (1, 41). Although HPV-18 has been less intensively studied than HPV-16, there is little doubt that HPV-18, too, plays a causative role in the development of cervical cancer (for a review, see reference 45).

Up to now, only limited information has been available about the humoral immune response to HPV-18 proteins in humans (22). In contrast, the presence of human antibodies to HPV-16 E2, E4, E7, L1, and L2 proteins has repeatedly been demonstrated by both Western blotting (immunoblotting) and enzyme-linked immunosorbent assay (ELISA) (8, 9, 24). Recently, we described a strong correlation between anti-E7 antibodies and cervical cancer by using bacterial fusion proteins in Western blot experiments (24). This test could be replaced by the less tedious ELISA, in which synthetic peptides of the antibody-reactive regions are used as antigens (29). In this paper, we report the identification of seroreactive regions within the HPV-18 E6 and E7 proteins and their use in identifying specific antibodies in human sera obtained from different geographic regions.

MATERIALS AND METHODS

HPV-18 fd phage expression library. The filamentous phage derivative fuse 1 (fd-tet-J6; 31, 37, 44) carrying the tetracycline resistance gene was used as an expression system for HPV-18 subgenomic DNA fragments cloned into its unique *Pvu*II site. DNA fragment insertions of the size $3n \pm 2$ nucleotides, without internal stop codons for translation, restore a frameshift mutation within gene III of bacteriophage fd which, in its mutated form, hinders the production of infectious progeny of nonrecombinant fuse 1 DNA.

The expression library was prepared by a protocol described earlier (29). In brief, 10 μg of HPV-18 DNA cloned at

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the single *Eco*RI site into pSP65 (28) was digested to a fragment size of about 200 bp by DNase I (Boehringer, Mannheim, Germany) in the presence of Mn^{2+}. To obtain blunt ends, we treated the DNA fragments with T4 polymerase and *Escherichia coli* DNA ligase (New England BioLabs GmbH, Schwabach, Germany) in the presence of the four deoxyribonucleotides. The DNA fragments were ligated into 1 μg of fuse 1 DNA at the unique *Pvu*II site. Cells of *E. coli* K802 (F⁻ *galK2 galT22 metB1 supE44 hsdr2*) (42) were transformed with the ligated DNA by the protocol of Hanahan (17) and plated on Luria broth (LB) plates containing 15 μg of tetracycline per ml. This method yielded a total of approximately 10^4 phage-producing colonies. A 100-fold-higher efficiency was obtained when cells of *E. coli* DH5α [F⁻ *supE44* Δ*lacU169* (ψ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (17) were transformed by electroporation (10). Each tetracycline-resistant colony produced identical bacteriophages which were not infectious for this *E. coli* F⁻ strain. From two independent ligations, a total of 1.2×10^5 tetracycline-resistant colonies were obtained; approximately 5% of these carried an HPV-18 subgenomic fragment of the right size and the correct orientation.

Antisera. Polyclonal rabbit antisera raised against an MS2 polymerase–HPV-18 E6 fusion protein or an MS2 polymerase–HPV-18 E7 fusion protein (34) were used. The virus-specific part of the HPV-18 E6 fusion protein was encoded by nucleotides at positions 132 to 591 (numbering according to Seedorf et al. [34]) and lacked the 4 N-terminal amino acids of the viral protein; the virus-specific part of the HPV-18 E7 fusion protein was encoded by nucleotides at positions 636 to 917 and did not include the 10 N-terminal amino acids of the viral protein (34). In addition, two monoclonal antibodies (MAbs), E6*/E6-18-1 (immunoglobulin G3) and E6*/E6-18-2 (immunoglobulin G1) (32), raised against an MS2 polymerase–HPV-18 E6* fusion protein were used.

Immune screening. Between 2,000 and 6,000 recombinant phages and 0.2 ml of exponentially growing *E. coli* K91 cells (F⁺ derivative of *E. coli* K38 [27]) in 3.5 ml of 0.5% agarose containing 10 mM $MgSO_4$ were overlaid on minimal agar plates. Nitrocellulose replicas were taken and incubated further on fresh minimal medium plates for 6 h at 37°C to enhance phage production. The filters were blocked for 60 min in 10% nonfat milk in phosphate-buffered saline (PBS) and incubated overnight in 5% nonfat milk in PBS containing 1:100 to 1:1,000 dilutions of HPV-specific rabbit antiserum (preabsorbed with sonicated K91 cells). The filters were washed five times for 5 min each time in PBS–0.1% Tween 20 and incubated for 3 h at room temperature with goat anti-rabbit peroxidase antibodies (1:1,000) in 5% nonfat milk. After being washed, the filters were stained in 50 ml of PBS containing 30 mg of diaminobenzidine, 30 μl of H_2O_2 (30%), and 1.5 ml of $NiSO_4$ (1%). Positive recombinant phages were picked and purified in several rounds of rescreening.

Preparation and sequencing of single-stranded DNA of fuse 1 recombinants. LB medium (50 ml) was inoculated with tetracycline-resistant *E. coli* K91 harboring recombinant fuse 1 plasmids. The culture was incubated overnight, and the bacteria were pelleted. After the addition of 2 ml of 40% polyethylene glycol 6000 and 2 ml of 5 M sodium acetate (pH 6.5) to the supernatant, the phages were precipitated for 60 min at 0°C and centrifuged at $3,000 \times g$ for 1 h. The pellet was resuspended in 10 mM Tris–0.1 mM EDTA (pH 8.1) buffer and, after two extractions with phenol, the DNA was precipitated. For sequencing, the standard United States Biochemicals (Cleveland, Ohio) protocol was used (primer: 5′ TCCAGACGTTAGTAAATGAA 3′).

Peptide pin synthesis. A set of overlapping decapeptides representing the HPV-18 E6 and E7 open reading frames (ORFs) were synthesized on the tips of polyethylene pins derivatized with β-alanine (Cambridge Research Biochemicals Ltd., Northwick, England) by the strategy described by Geysen et al. (15, 16). The viral protein sequence was divided into decamer peptides overlapping each other by eight amino acids. Synthesis was carried out with 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, and in situ activation was carried out with benzotriazolyl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP; Castro's reagent) (3). Fmoc amino acid derivatives (10 μmol), BOP (10 μmol), and *N*-methyl morpholine (20 μmol) solutions were distributed into polyethylene reaction trays (Cambridge Research Biochemicals) on the basis of the respective peptide sequence being synthesized. All other reactions were carried out in accordance with the manufacturer's instructions. As a positive control, the peptide RPDYLD FAAA, derived from human tumor necrosis factor alpha (4), was synthesized together with the HPV-18-specific peptides and tested with an appropriate antiserum by ELISA.

Peptide pin screening. For epitope mapping, tests were carried out with peptides bound covalently to the polyethylene pins on which they had originally been synthesized. The pins were blocked with PBS–1% bovine serum albumin–0.05% Tween 20 for 1 h at 37°C and incubated with sera diluted 1:50 to 1:4,000 in PBS–1% bovine serum albumin–0.05% Tween 20. After being washed with PBS–0.05% Tween 20, the pins were incubated for 1 h at 37°C with protein A-peroxidase (1:4,000) and washed again. Staining was performed with tetramethylbenzidine (18) for 10 min and terminated by removing the pins from the dye and adding 100 μl of 0.5 M H_2SO_4. The extinction was measured in a Titertek ELISA reader at 450 nm (E_{450}).

To remove the antibody-enzyme complex after peptide pin screening, we sonicated the pins for 1 h (30 W, 48 kHz) in H_2O–1% sodium dodecyl sulfate–0.1 M NaH_2PO_4–0.1% 2-mercaptoethanol (pH 7.2) at 60°C and washed them with methanol and water. The efficiency of the disruption procedure was determined by peptide pin screening with protein A-peroxidase but without primary serum. The same peptides were used more than 30 times in subsequent peptide pin ELISAs.

Synthesis of peptides for ELISA. Peptides (pepE7/1, pepE6/1, pepE6/2, pepE6/3, and pepE6/4) were synthesized in a continuous-flow instrument constructed and operated as described earlier (11). Peptide chain assembly was performed by the solid-phase method on a 1% cross-linked polystyrene support with Fmoc amino acids and in situ activation by BOP.

Peptide pepE701 was assembled on PepSyn KA resin in a Milligen/Biosearch model 9050 automated peptide synthesizer with pentafluorophenyl esters of Fmoc amino acids plus 1-hydroxybenzotriazole (HOBt) (for serine, the 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine ester was used; for arginine, the BOP-HOBt method was used). Protecting groups were as follows: *t*-butyl ester for Glu and Asp, *t*-butyloxycarbonyl for His, *t*-butyl for Ser, and methoxytrimethylbenzene sulfonyl for Arg. The peptide chain was cleaved from the resin, and the protecting groups were removed with trifluoroacetic acid-ethanedithiol-thioanisole-anisole (90:3:5:2).

The synthetic peptides were deprotected, cleaved from the solid support by treatment with trifluoroacetic acid, and

purified by reversed-phase high-pressure liquid chromatography.

Human sera. A total of 116 serum samples collected from patients (age range, 15 to 70 years; mean age, 45 years) with invasive squamous cell carcinoma of the uterine cervix at the Tanzania Tumor Centre in Dar es Salaam, Tanzania, were tested in the ELISA described below. Sera from gynecological in-patients attending the Muhimbili Medical Centre in Dar es Salaam, Tanzania, for other gynecological problems, such as uterine fibroid tumors, pelvic inflammatory disease, or pregnancy complications, were tested as controls. For each tumor patient, an age-matched female patient (with a maximum age difference of 5 years; age range, 15 to 68 years; mean age, 43 years) was tested.

Serum samples collected from 94 patients (age range, 23 to 84 years; mean age, 58.3 years) with invasive squamous cell carcinoma of the uterine cervix at the Gynecology Hospital, University of Düsseldorf, Düsseldorf, Germany, and an equal number of serum samples collected from age-matched female controls (maximum age difference, 5 years; age range, 25 to 82 years; mean age, 55.5 years) in the same institution were tested. In most of these control patients, breast cancer had been diagnosed.

ELISA with human sera. Each well of 96-well microtiter plates (Dynatech catalog no. 001-010-2401) was coated overnight at room temperature with 50 μl of 10-μg/ml (pepE6/2; see Results) or 25-μg/ml (pepE7/1, pepE701, pepE6/1, pepE6/3, and pepE6/4; see Results) synthetic peptide solutions in PBS or 0.06 M sodium carbonate buffer (pH 9.6) (depending on the coating properties of the peptide). After being washed three times with water, the plates were blocked with 0.2% gelatin (Merck, Darmstadt, Germany) in PBS for more than 3 h at 37°C, washed once with PBS–0.05% Tween 20, and incubated with human sera diluted 1:100 (unless indicated otherwise) in PBS–0.2% gelatin–0.05% Tween 20 for 1 h at 37°C. The plates were washed five times and incubated with a 1:25,000 dilution of a goat anti-human peroxidase antibody (anti-immunoglobulin G; Zymed Laboratories Inc., San Francisco, Calif.) in PBS–0.2% gelatin–0.05% Tween 20 for 1 h at 37°C. After being washed eight times, the plates were incubated with 2,2′-azinobis(3-ethylbenzthiazolinium-6-sulfonate) at 1 mg/ml–0.01% H_2O_2–0.1 M sodium acetate (pH 4.2) for 60 min before the E_{405} was measured in a Titertek ELISA reader. To achieve comparable conditions, we tested identical numbers of case and control sera on the same plate. Each human serum sample was tested twice in a peptide-coated well and in a well with no added peptide. The reactivity of a serum sample was calculated from the difference between the mean of the duplicate extinction values for the peptide-containing wells and the mean of the duplicate blank extinction values (for the wells with no added peptide). Blank E_{405} values ranged from 0.02 to 0.2. Reactivity values were analyzed by statistical methods that included a cutoff point (asymptotic Fisher test) (14) or considered the distribution as a whole without defining a cutoff value (mixing distribution test) (6).

Competition assay. A competition assay was performed by incubating human sera in PBS (final dilution, 1:100) with various amounts of infectious particles of the recombinant phage preparation for 2 h at 37°C. After centrifugation in a Sigma centrifuge 2 MK at 13,000 rpm for 20 min, the supernatant was tested in the ELISA as described above. Recombinant phages were prepared as described above. The pellet was resuspended in 200 μl of TE (Tris-EDTA), and the phage titer was determined by plating on *E. coli* K91 cells. Approximately 10^9 infectious particles per μl were found in all preparations made from a 200-ml culture.

RESULTS

Identification of antibody-reactive regions on HPV-18 E6 and E7 proteins. Seroreactive regions were identified by two independent methods: immune screening of a bacteriophage expression library and testing of overlapping decapeptides covalently bound to polyethylene pins.

Approximately 75,000 recombinant fd bacteriophages from two separately constructed HPV-18 DNA libraries were plated on *E. coli* cells, and reactive plaques were detected by immune screening as described by Müller et al. (29). Thirty clones of bacteriophages reacting with polyclonal rabbit antisera raised against MS2 polymerase–HPV-18 E6 or E7 fusion proteins were obtained after repeated rescreening and subsequent purification; 16 of these were further characterized. Single-stranded DNA from bacteriophage particles was used for sequencing the insert.

The polyclonal anti-HPV-18 E6 rabbit serum detected two groups of bacteriophages (ph 155 and ph 136), each carrying identical inserts of viral DNA. The insert of bacteriophage ph 155 (HNTMARFEDPTRRPYKLPDLCTELNTSLQDIE ITCVYCKT; four identical isolates) comprised the first 37 amino acids of the E6 ORF plus 3 amino acids derived from the 3′ part of the upstream regulatory region (Fig. 1). Two identical clones containing the ph 136 insert (ACHKCIDF YSRIRELRHYSDSVYGDTLEKLT; amino acid positions 64 to 94) were isolated.

The serum generated against an HPV-18 E7 fusion protein reacted with two groups of recombinants (ph 20 obtained in two and ph 307 obtained in eight identical isolates) with overlapping inserts: ph 307 contained the 29-amino-acid insert QLSDSEEENDEIDGVNHQHLPARRAEPQR, representing the C-terminal part of the 47-amino-acid insert, VLHLEPQNEIPVDLLCHEQLSDSEEENDEIDGVNHQH LPARRAEPQR, of ph 20 (amino acid positions 12 to 58) (Fig. 2).

As an alternative method for identifying antibody-reactive regions on the HPV-18 E6 and E7 proteins, overlapping decapeptides representing the HPV-18 E6 (75 peptides) and E7 (48 peptides) ORFs were synthesized on polyethylene pins and tested with various sera (peptide pin screening). Each peptide overlapped the following one by 8 amino acids, e.g., peptide 1 spanning amino acids 1 to 10 overlapped peptide 2 spanning amino acids 3 to 12.

The decapeptides representing the HPV-18 E6 ORF were tested with the polyclonal rabbit antiserum raised against an MS2 polymerase–HPV-18 E6 fusion protein, and four reactive regions were identified (Fig. 1). The two N-terminal clusters of reactive peptides, designated the E6/1 and E6/2 regions, corresponded to the inserts of reactive bacteriophages ph 155 and ph 136 isolated by screening of the fd expression library as described above. Two MAbs raised against an MS2 polymerase–HPV-18 E6* fusion protein (32) were also tested by peptide pin screening. The HPV-18 E6* protein shares 43 N-terminal amino acids with the E6 protein (33). Both MAbs showed strong reactivity with the four N-terminal decapeptides corresponding to the E6/1 epitope and failed to react with the remaining peptides (Fig. 3).

The overlapping peptides representing the HPV-18 E7 ORF were tested with the polyclonal rabbit antiserum raised against an MS2 polymerase–HPV-18 E7 fusion protein. A single reactive region comprising peptides 20 to 26 was found and was designated the E7/1 region (Fig. 2). This localization

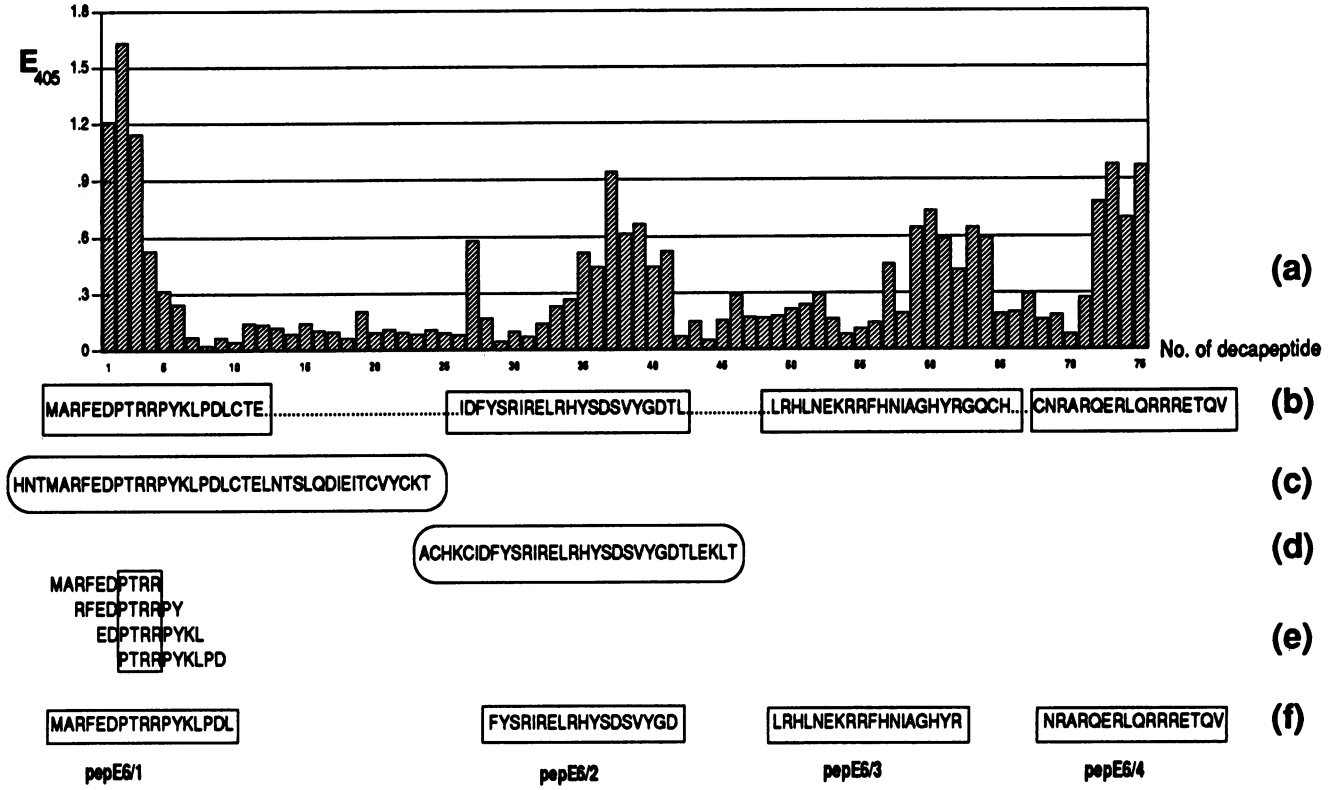

FIG. 1. Immunoreactive regions on the HPV-18 E6 protein. (a) Peptide pin screening of 75 overlapping decapeptides representing the HPV-18 E6 ORF with the polyclonal rabbit antiserum anti-E6. (b) Delineation of the reactive regions found by this approach. (c) Insert of recombinant fd phage 155. (d) Insert of recombinant fd phage ph 136. (e) Four N-terminal decapeptides recognized by MAbs E6*/E6-18-1 and E6*/E6-18-2 (see Fig. 3); the four amino acids present in all four peptides are boxed. (f) Sequences of the synthetic peptides used in the ELISA.

corresponded to the inserts of ph 20 and ph 307 isolated by screening of the fd expression library with the same polyclonal rabbit antiserum. Peptide pin screening was also applied to human sera collected in Dar es Salaam, Tanzania. For this purpose, positive and negative human sera were identified by ELISA with peptides pepE7/1 and pepE701 (see below; Fig. 2). When compared with an ELISA-negative serum, sample 90/2/40 showed a significant increase in extinction with peptides 23 to 25, whereas a group of nine serum samples (e.g., sample 90/1/63) clearly reacted with peptides 4, 19, and 20 (Fig. 2). This observation indicates the existence of two adjacent epitopes which are located at peptides 19 and 20 and peptides 23 to 25 and which are recognized by sera of different individuals. It is not clear whether the reactivity with peptide 4 can be considered specific, as the neighboring overlapping peptides were negative. On the other hand, the corresponding area of the HPV-16 E7 protein was described as an antigenic epitope in mice (40).

Detection of HPV-18-specific antibodies by ELISA. On the basis of the HPV-18 E6- and E7-seroreactive regions delineated above, an ELISA protocol was established with the following synthetic peptides as targets on microtiter plates: pepE7/1 (IDGVNHQHLPARRAEPQR), pepE701 (SDSEE ENDEIDGVNHQHLPARRAEPQRH), pepE6/1 (MARFEDP TRRPYKLPDL), pepE6/2 (FYSRIRELRHYSDSVYGD), pepE6/3 (LRHLNEKRRFHNIAGHYR), and pepE6/4 (NR ARQERLQRRRETQV). These peptides were recognized by polyclonal rabbit antisera prepared against MS2 polymerase–HPV-18 E6 and E7 fusion proteins (data not shown), demonstrating that the peptides coated on the microtiter wells were presented in a conformation that allowed a specific antigen-antibody reaction.

Serum samples collected from 116 human patients with invasive squamous cell carcinoma of the uterine cervix at the Tanzania Tumor Centre and 116 serum samples collected from patients attending the Muhimbili Medical Centre in Dar es Salaam, Tanzania, for reasons other than cervical cancer were tested in the HPV-18 ELISA.

With the four HPV-18 E6 peptides, no difference in reactivity between the two groups of patient sera was observed, defining a positive test as an E_{405} of >0.2. Only one human serum sample (90/2/40), belonging to the cancer group, recognized the pepE6/1 peptide. In addition, this serum sample was positive with the pepE7/1 and pepE701 peptides. Five human serum samples reacting with the pepE6/2 peptide were detected in each group. No human serum sample reacted with the pepE6/3 peptide. Three serum samples from the case group and one serum sample from the control group were positive with the pepE6/4 peptide.

When the pepE701 peptide, which comprises both reactive epitopes located close to each other on the HPV-18 E7 protein, was used, marked differences were found between cases and controls (Fig. 4). Ten of the 116 serum samples collected from carcinoma patients reacted with the pepE701 peptide, while no serum sample from the control group showed an E_{405} of >0.15. When the pepE7/1 peptide, which

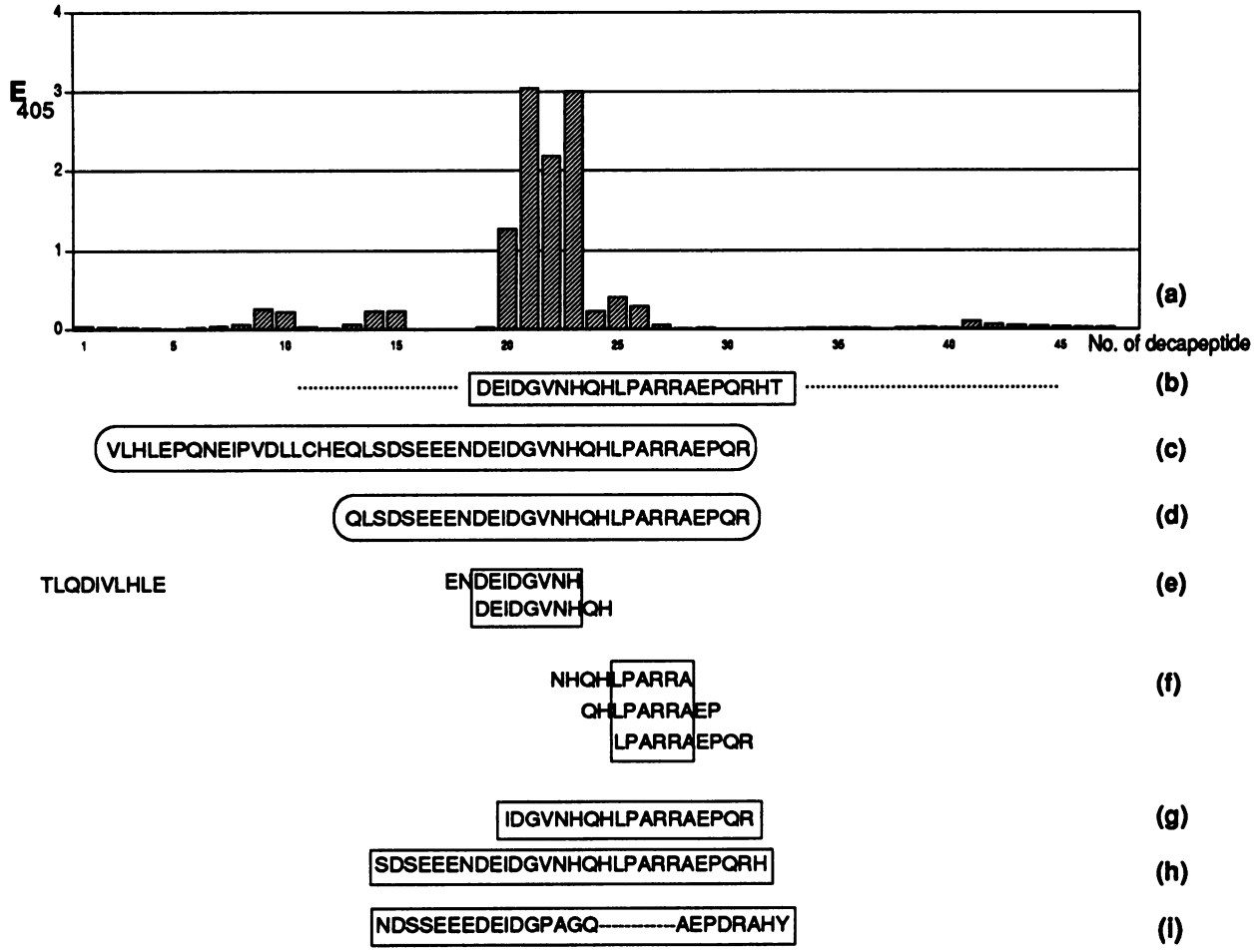

FIG. 2. Immunoreactive regions on the HPV-18 E7 protein. (a) Peptide pin screening of 48 overlapping decapeptides representing the HPV-18 E7 ORF with the polyclonal rabbit antiserum anti-E7. (b) Reactive region comprising peptides 20 to 26. (c) Insert of recombinant fd phage ph 20. (d) Insert of recombinant fd phage ph 307. (e) Peptides 4, 19, and 20 recognized by human serum 90/1/63; the overlapping region is boxed. (f) Peptides 23, 24, and 25 (the overlapping hexapeptide is marked) recognized by human serum 90/2/40. (g) Sequence of the synthetic peptide pepE7/1 used in the ELISA. (h) Sequence of the synthetic peptide pepE701 used in the ELISA. (i) Peptide representing the immunoreactive region E7-107 of HPV-16 described earlier (29); identical amino acids are marked.

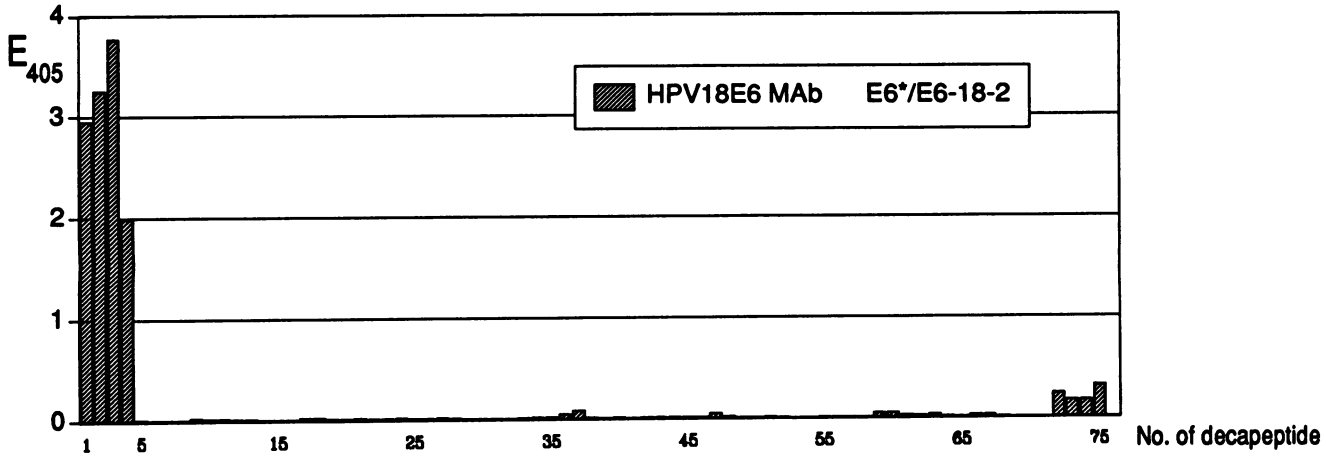

FIG. 3. Reactivity of MAb E6*/E6-18-2 with the 76 overlapping peptides representing the HPV-18 E6 ORF. The same epitope was recognized by MAb E6*/E6-18-1. This reactive region is referred to as the E6/1 epitope.

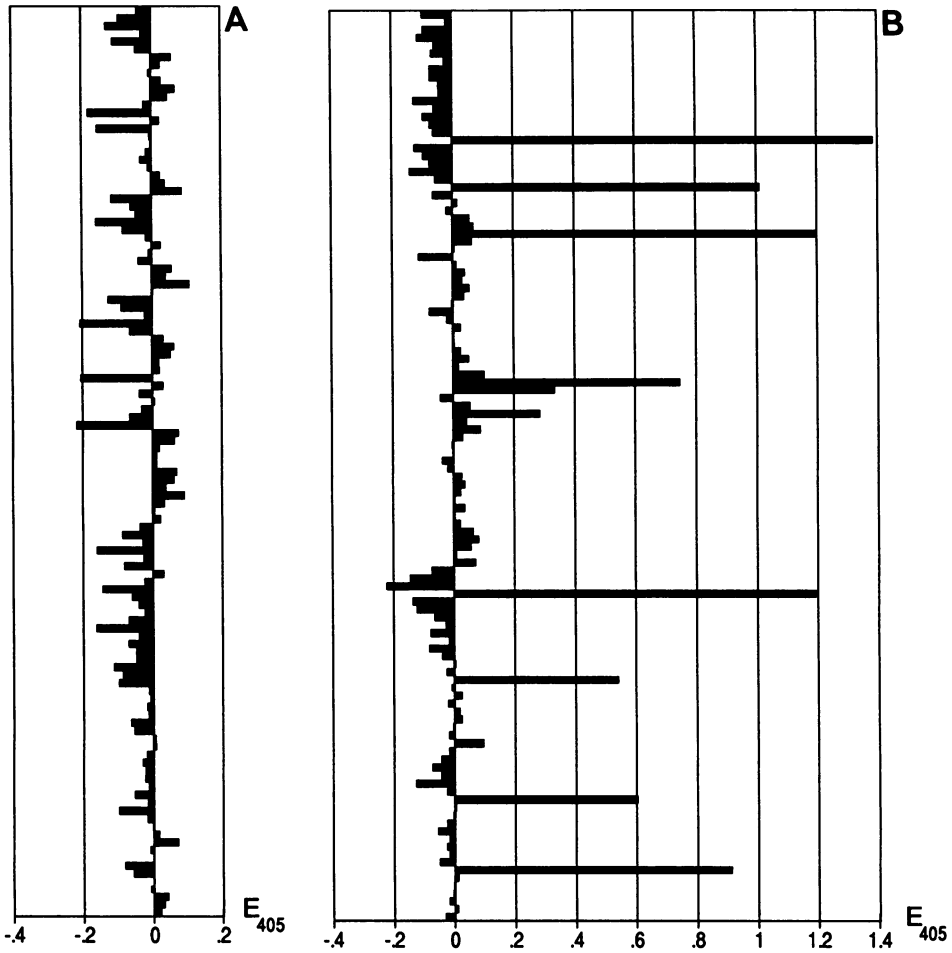

FIG. 4. pepE701 reactivities of 116 control serum samples (A) and 116 serum samples from patients with invasive carcinoma of the uterine cervix (B), collected in Dar es Salaam, Tanzania. Each bar displays the extinction value (E_{405}) for one serum sample. Negative bars resulted from blank values higher than the peptide value.

contains only the C-terminal epitope of the two adjacent epitopes, was used, only one human serum sample (90/2/40), belonging to the case group, was positive. For the pepE701 ELISA, the difference in the extinction values between the case and control groups was highly significant, as determined by the asymptotic Fisher test (14) ($P < 0.001$ for a cutoff point of $E_{405} = 0.15$ or $P = 0.002$ for a cutoff point of $E_{405} = 0.1$) and the mixing distribution test ($P = 0.009$) (6).

Of 94 serum samples collected from cervical cancer patients at the Gynecology Hospital, University of Düsseldorf, Düsseldorf, Germany, 3 were found to be positive in the ELISA with the pepE701 peptide, while 0 of 94 control serum samples generated an E_{405} of >0.15 (Fig. 5). The number of positive samples did not increase when the serum samples were tested at a 1:25 dilution instead of the routinely used 1:100 dilution. The higher prevalence of anti-HPV-18 E7 antibodies in Tanzanian samples than in German samples coincides with an increased proportion of HPV-18 DNA-positive cervical carcinomas in Africa (2, 5, 7, 20, 39).

Specificity of the detected antibodies. To confirm the specificity of the reactions of the human sera in the peptide ELISA, we performed competition assays. Bacteriophage preparations of recombinant fd phages containing specific inserts were used to block the reactions of human antibodies to peptides. A recombinant bacteriophage with no insert was used as a control. Phage particles carrying the insert of ph 307 were able to suppress the specific reaction of serum sample 90/2/40 (1:100) to the pepE701 peptide in a concentration-dependent manner (Fig. 6). The same number of infectious fd phage particles without the insert did not suppress the reaction. The positive signal of the same serum sample with the pepE6/1 peptide was not reduced by phage ph 307, demonstrating that the phage preparation did not inactivate the antibodies nonspecifically. The specificity of the reaction of serum sample 90/2/40 with the pepE6/1 peptide was demonstrated in the same manner with bacteriophage ph 155 (data not shown).

To define the type specificity of the peptide ELISA, we analyzed tumor biopsies of 24 Tanzanian patients for the presence of HPV DNA by Southern blotting or the polymerase chain reaction (39) and correlated the virologic data with the detection of antibodies. Eight of the cancer biopsies were found positive for HPV-18 DNA, and four of the respective serum samples gave an E_{405} of >0.1 in the ELISA with the pepE701 peptide (mean value, 0.365). In contrast, none of the serum samples from patients whose biopsies contained HPV-16 (13 patients) or HPV-45 (1 patient) or were devoid of detectable HPV DNA (2 patients) gave an E_{405} of >0.1.

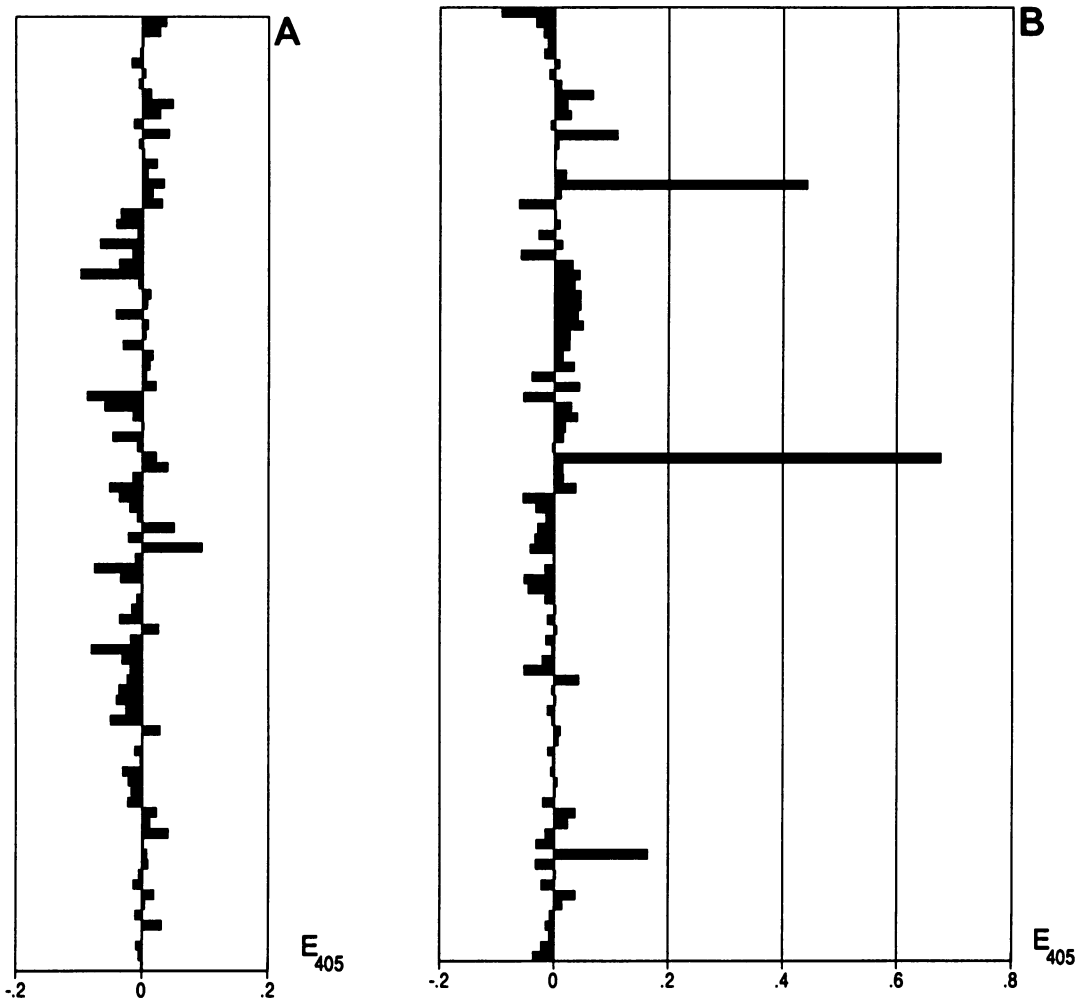

FIG. 5. pepE701 reactivities of 94 control serum samples (A) and 94 serum samples from patients with invasive carcinoma of the uterine cervix (B), collected in Düsseldorf, Germany. Each bar displays the extinction value (E_{405}) for one serum sample. The ELISA protocol was modified with a 1:25 dilution of the sera (see the text).

All 10 human serum samples from Tanzania that reacted with the HPV-18 pepE701 peptide were negative in ELISAs with the peptides representing the two previously identified antibody-reactive regions within the HPV-16 E7 protein (29), although the HPV-16 E7-107 region (29) shares approximately 60% amino acid homology with the HPV-18 E7 epitope (Fig. 2). Conversely, seven serum samples positive in ELISAs with the HPV-16 E7 peptides were negative when tested in an HPV-18 pepE701 peptide ELISA.

DISCUSSION

We have identified seroreactive regions on the HPV-18 E6 and E7 proteins with rabbit polyclonal antisera by two different techniques: immune screening of an HPV-18 bacteriophage fd expression library and testing of overlapping peptides (peptide pin screening).

Of the four reactive regions on the E6 protein (E6/1 to E6/4) found by peptide pin screening, two (E6/1 and E6/2) were also represented in HPV-18 fd recombinant bacteriophages. Region E6/1 was also recognized by MAbs raised against the HPV-18 E6* protein (32) (Fig. 1d). These MAbs also react with the native E6 protein (32), which is identical to the E6* protein in the N-terminal part (33). In addition, all but one region (E6/3) reacted with a small number of human sera when synthetic peptides comprising these regions were used in the ELISA. It remains to be elucidated whether additional regions which react with human sera but were missed by our approach with rabbit hyperimmune serum exist within the HPV-18 E6 protein.

In the HPV-18 E7 ORF, one reactive region encompassing 20 amino acids was found (Fig. 2). Testing the peptide pins with different human sera, however, demonstrated the presence of two separate epitopes within this region. The N-terminal epitope was recognized more frequently. This observation was confirmed when the two peptides, pepE7/1 and pepE701 (Fig. 2g and h), were used to test 232 human serum samples in an ELISA. Ten serum samples were positive with pepE701, but only 1 serum sample reacted with pepE7/1, which contains only the C-terminal epitope.

Our results demonstrated that human sera as well as polyclonal antibodies MAbs produced by immunization with denatured fusion proteins reacted with the same region of the HPV-18 E7 protein. This result indicates that this region is presented to the immune system in the context of the authentic protein, too. The capability of the fusion protein to generate an immune response which resembles that in hu-

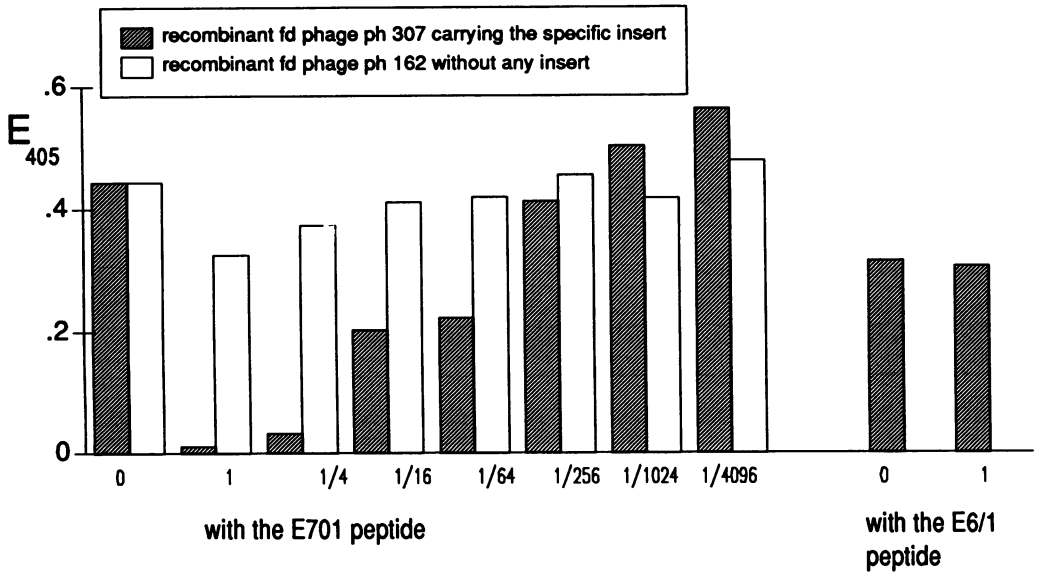

FIG. 6. Competition assay with human serum sample 90/2/40 in a 1:100 dilution blocked by various amounts of recombinant phage particles. ph 307 is the fd recombinant containing the specific insert; ph 162 is the fd recombinant containing no insert. Extinction values were detected with pepE701 and pepE6/1, respectively. 0, without phage block; 1, 1.6×10^{11} infectious particles; 1/4, 4×10^{10} infectious particles, etc.

mans underlines the validity of serological tests that use bacterial fusion proteins (19, 21, 24).

Fifteen of 232 human sera reacted with the HPV-18 E6 peptides pepE6/1, pepE6/2, and pepE6/4. No difference in prevalence, however, between cervical cancer patients and age-matched controls was found. The occurrence of anti-HPV-18 E6 antibodies in the course of a papillomavirus infection remains to be elucidated.

Confirming earlier results obtained by Western blotting with HPV-16 E7 fusion proteins (24), there was a strong correlation between anti-HPV-18 E7 seroreactivity and the presence of cervical cancer: 13 of 210 tumor patient serum samples but 0 of 210 control serum samples were positive. The difference in prevalence was even more pronounced when only sera collected in Tanzania were compared (10 of 116 versus 0 of 116; Fig. 4). A low prevalence of anti-HPV-18 E7 antibodies was also detected by Western blotting in cervical cancer patient sera collected in the United States and in Germany (13, 23). The higher proportion of HPV-18 E7-positive sera obtained from African patients may reflect the different prevalences of HPV-18 DNA-positive cervical cancer biopsies in Western countries and in Africa (2, 5, 7, 20, 39). A preferential association of HPV-18 with adenocarcinomas has been reported (25, 38, 41). It will be interesting to see whether antibodies to E7 can be found more frequently in such patients.

A total of 24 tumor samples from the Tanzanian patients were available for DNA analysis, and HPV-18 genomes could be detected in 8 of these (39). Serum samples obtained from four of these patients contained antibodies which reacted with the pepE701 peptide. A similar proportion of samples positive for E7 was found when serum samples from patients with HPV-16 DNA-positive cervical cancer were tested in peptide ELISAs with HPV-16 E7-reactive regions (29, 30). It is not clear why only a fraction of HPV DNA-positive persons have sera which react with E7 peptides in an ELISA. Some theories are as follows. (i) In the native protein, additional linear and also conformational epitopes may exist, the latter of which cannot be identified by the methods used in this study and in a previous study (29). In fact, Selvey and colleagues recently reported evidence for the presence of E7-specific conformational epitopes when mouse MAbs established against the MS2 polymerase–HPV-18 E7 fusion protein were tested (35). (ii) Anti-E7 antibodies may develop only late in tumor progression. A possible correlation between seropositivity and tumor staging is currently under investigation in our laboratory. (iii) HPV variants which differ in the seroreactive region of the E7 protein may be present in the population. If such mutations within individual HPV-18 isolates can be identified, we intend to use the correspondingly modified peptides to test sera from HPV-18 DNA-positive cervical cancer patients which failed to react with the "wild-type" E7 epitope. In general, it remains to be tested whether additional cervical cancer patients can be found positive for anti-E7 antibodies by the Western blot technique.

In addition to the correlation of anti-E7 antibody positivity with the presence of HPV-18 DNA in cervical cancers, the type specificity of the anti-HPV-18 E7 antibodies was also demonstrated by the failure of HPV-18-positive sera to react with the homologous HPV-16 E7 region. Conversely, sera reactive with the HPV-16 E7 epitope gave negative results when tested in the HPV-18 E7 peptide ELISA. Similar observations were made when HPV-16 and HPV-18 E7 fusion proteins were tested by Western blotting (22, 23). The type specificity seen in the ELISA is particularly remarkable, since the respective regions differed in only a few amino acids, especially in the more frequently reactive N-terminal portion of the pepE701 peptide (Fig. 2e, h, and i). From this observation, it is anticipated that the homologous regions of HPV types other than 16 and 18 may also represent seroreactive domains. Therefore, in future experiments such regions will preferentially be tested for their ability to bind specific antibodies.

As also shown for HPV-16 (24), antibodies to the E7 protein are rarely found in healthy persons and thus cannot

be considered a diagnostic marker for virus infection. Therefore, epitopes of other viral proteins need to be identified to assess the prevalence and natural history of HPV-18 infections. In contrast, anti-E7 antibodies are detected with greater frequency in sera obtained from cervical cancer patients. Therefore, the immune system seems to respond to the constant exposure to the E7 protein in persistently infected cells. Future studies should investigate whether the antibodies appear prior to the clinical diagnosis of cervical cancer or whether they represent a marker for tumor progression. In addition, the immunoreactivity to other early papillomavirus proteins must be analyzed to obtain a comprehensive picture of the correlation between the anti-HPV response and clinical disease. A detailed knowledge of the immune response to HPV infection is a prerequisite to designing a strategy for the development of an antiviral vaccine or antitumor immune therapy.

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